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RELATIVE ABUNDANCE OF TREE HOLE–BREEDING MOSQUITOES IN BOONE COUNTY, MISSOURI, USA, WITH EMPHASIS ON THE VECTOR POTENTIAL OF *AEDES TRISERIATUS* FOR CANINE HEARTWORM, *DIROFILARIA IMMITIS* (SPIRURIDA: FILARIIDAE)[1]

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ABSTRACT. *Aedes* (*Protomacleaya*) *triseriatus* currently shares its habitat in the USA with the introduced species *Aedes* (*Finlaya*) *japonicus* and *Aedes* (*Stegomyia*) *albopictus*. In the late 1980s, before the introduction of these 2 species, *Ae. triseriatus* was the dominant tree hole– and artificial container–breeding mosquito in central Missouri. *Aedes triseriatus* represented 89% of the mosquito immatures collected from water-filled tree holes and artificial containers at 3 forested field sites in central Missouri, from May to October, 1986 to 1988. Laboratory-reared female *Ae. triseriatus* were able to support larval development of *Dirofilaria immitis* (canine heartworm) to the infective 3rd larval stage. A blood meal from a microfilaremic Collie-mix dog was sufficient to infect adult female mosquitoes, indicating that *Ae. triseriatus* is a possible vector of canine heartworm in central Missouri. Confirmation of the vector status of this species depends on the yet-to-be observed transmission of *D. immitis* by *Ae. triseriatus* in the field, possibly by experimental infection of dogs by wild-caught mosquitoes. Defining the role of this species in epizootic outbreaks could contribute toward accurate risk assessment as the abundance of *Ae. triseriatus* increases and decreases in response to the success of *Ae. albopictus*, *Ae. japonicus*, or other introduced container-breeding mosquitoes.

KEY WORDS *Aedes triseriatus*, *Dirofilaria immitis*, tree hole mosquito, container-breeding mosquito, vector potential, invasive species

INTRODUCTION

Canine dirofilariasis, or dog heartworm, is an important disease of dogs and has progressively spread throughout the USA (Graham 1974; Otto 1975, 1986; Myahara et al. 1976; Sears et al. 1980; Nzabanita et al. 1982; Walters and Lavoipierre 1984; Nayar 1998). Although canine infection results in severe disease that often progresses to a fatal syndrome, rare human infections are limited to focal pulmonary infection with granuloma formation (Merrill et al. 1980). Human infection with canine dirofilariae has been reported in the USA (Shah 1999), Japan (Yoshimura et al. 1980), Italy (Pampiglione et al. 1991), and Taiwan (Tsung and Liu 2003). In the USA, many cases are not reported, either because the symptoms are not serious enough to arouse concern or because the disease is misdiagnosed. In Missouri, the prevalence of canine dirofilariasis is an increasing problem of domestic dogs, and it occurs in 4–10% of all dogs sampled (Pratt et al. 1981, Pratt and Corwin 1984).

Little information is available on the mosquito vectors of canine heartworm, *Dirofilaria immitis* Leidy, in Missouri (Smith 1967). In an attempt to determine the potential of a tree hole–breeding mosquito, *Aedes* (*Protomacleaya*) *triseriatus* (Say), as a vector of *D. immitis* in Missouri, we performed a 2-phased study from 1986 to 1988. The 1st phase was a survey of tree holes and artificial containers aimed to assess the abundance of the mosquito species in those habitats. The 2nd phase was a laboratory study to determine vector competence of *Ae. triseriatus* for *D. immitis*. Our results provide relevant baseline information about the tree hole–breeding mosquito fauna in Missouri. This baseline is particularly relevant now because of the subsequent introduction of *Ae.* (*Finlaya*) *japonicus* (Theobald), a potential vector of West Nile virus (WNV) and *Ae.* (*Stegomyia*) *albopictus* (Skuse), a potential vector of dengue and WNV.

MATERIALS AND METHODS

Study sites: Three forested field sites (Hearnes, Hinkson Bottoms, and Ashland Wildlife Reserve) in Boone County, Missouri (38°57′N, 92°20′W) were used to study the tree hole– and artificial container–breeding mosquito *Ae. triseriatus* from May to October in each year of 1986 through 1988. The dominant trees in the 3 sites were yellow chestnut oak (*Quercus muhlenbergii* Engelm.), green ash (*Fraxinus pennsylvanica* Marsh.), American elm (*Ulmus americana* Linn.), box elder (*Acer negundo* (Linn.)), white oak (*Quercus alba* Linn.), and silver maple (*Acer saccharinum* Linn.).

Sampling techniques and identification: Mosquito larvae and pupae were sampled weekly from permanently labeled tree holes and artificial containers, and the mosquitoes were transported to the labo-

[1] This article reports the results of research only and the opinions expressed herein are those of the authors and do not reflect those of the University of Missouri–Columbia or the U.S. Department of Defense.

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ratory for rearing and identification. Three replicate samples per site on each sampling occasion were obtained by using a 60-ml wide-mouthed pipette. The water temperature of each tree hole or artificial container was determined at the time of sampling and the pH and electrical conductivity were determined in the laboratory using a Fisher Accument pH Meter, Model 144 and a Markson Model 4503 Selectro Mark Analyzer equipped with a conductivity meter (Lab Extreme, Inc, Kent City, MI).

In this paper, the use of composite genus *Aedes* follows that of Knight and Stone (1977) instead of Reinert (2000). We also chose to retain Edward's generic designation of *Aedes* (Savage and Strickman 2004), especially considering recent analyses of *Ochlerotatus* indicating its polyphyly as a genus (Reinert et al. 2004). Specimens were identified to species by using the keys of Darsie and Ward (1981).

Mosquito hosts: The development of canine heartworm was studied in adult *Ae. triseriatus* reared from field-collected larvae and pupae. The mosquito immatures (larvae and pupae) collected from tree holes and artificial containers were transferred in the laboratory to plastic rearing cups (4.5 cm diameter × 3.8 cm deep) with 30 ml of distilled water, 20 mg of a dried mixture (1:1 volume) of yeast and Purina Rabbit Chow (Purina Mills, LLC, St. Louis, MO), and 0.5 g of leaf litter (Chambers 1985). These containers were placed on white enamel rearing trays (35 × 18 × 5.6 cm) and reared at room temperature (ca. 22.5°C). The water surface was skimmed regularly with a paper towel to minimize larval mortality caused by scum on the water surface. Field-collected and laboratory-reared pupae were transferred to plastic containers filled with distilled water (20 pupae/100-ml container). The pupae were held in a cage (30.5 cm on a side) at ca. 25°C and 70–80% relative humidity in a rearing room. As many as 200 pupae were placed in each of the cages. Cotton balls saturated with a 10% sucrose solution were constantly available to emerging adults.

Infection of Ae. triseriatus*:* The laboratory-reared female mosquitoes were infected with microfilariae of *D. immitis* by 2 different methods. During the 1st phase of the study in the 1986 summer season, the source of infection was blood from a dog infected with *D. immitis*. Four 5-ml blood samples were taken in a heparinized syringe from the radial vein of the forelimb. These samples were subsequently put into a test tube containing ethylenediaminetetraacetic acid (EDTA) at 1.5 mg/ml as an anticoagulant. The blood was then provided to female mosquitoes through a membrane on blood-soaked cotton pledgets. This method was eventually discontinued because of the high mortality of female mosquitoes (data not shown). In the 2nd phase of the study in the 1987 and 1988 summer seasons, the source of infection was blood taken directly by mosquitoes from a dog (Collie mixed breed) naturally infected with *D. immitis*. The dog was maintained in a mosquito-proof, indoor kennel at the Department of Laboratory Animal Medicine, University of Missouri–Columbia.

Samples of blood were processed by the modified Knott's test (Knott 1939) to make counts of *D. immitis*. Female mosquitoes to be infected were starved 2 days after emergence before feeding on the dog. The dog was anesthetized with 5% sodium thiopental (1 ml/2 kg body weight) and its hind limb was shaved with an electric livestock shaver before inserting the leg into the rearing cage through the access sleeve. The female mosquitoes were then allowed to feed to repletion (ca. 30–45 min). After the infective blood meal, the cage(s) containing the mosquitoes were transported to a rearing room (ca. 22.5°C). Mosquitoes were given access to cotton balls soaked with 10% sucrose solution on petri dishes inside the cages as a supplemental food source for the mosquitoes during the developmental period of *D. immitis*.

Evaluation of larval development of D. immitis*:* Bloodfed female mosquitoes were dissected at intervals after infection to determine the development of the infective 3rd larval stage of microfilariae of *D. immitis*. The dissection procedure was as follows. The female mosquitoes were cut into 3 major parts (abdomen, head, and thorax) with fine, thin-tip forceps under a dissecting microscope in a mixture of 10% glycerol, 1% Tween 80, and distilled water. A coverslip was then placed over the dissected body section, which was examined under 40× magnification with a compound microscope to check for the presence of developing larval forms of *D. immitis*. Female mosquitoes were dissected at the following intervals after the infective blood meal: 2 days (5 mosquitoes), 9 days (10 mosquitoes), 13 days (15 mosquitoes), 15 days (20 mosquitoes), and 17 days (25 mosquitoes).

Statistical analyses: Data generated from this study were tested by analysis of variance (SAS Institute 1985).

RESULTS

Eight species of mosquitoes in 6 genera were collected from water-filled tree holes and artificial containers at 3 study sites in Boone County, Missouri, from May to October, 1986 to 1988 (Table 1). They included *Anopheles* (*Anopheles*) *barberi* Coquillett, *Culex* (*Culex*) *restuans* Theobald, *Cx.* (*Neoculex*) *territans* Walker, *Ae.* (*Pro.*) *triseriatus* (Say), *Ae.* (*Pro.*) *hendersoni* (Cockerell), *Orthopodomyia signifera* (Coquillett), *Psorophora* (*Janthinosoma*) *ferox* (Von Humboldt), and *Toxorhynchites* (*Lynchiella*) *rutilus septentrionalis* Dyar and Knab. *Aedes triseriatus* was the most abundant tree hole– and artificial container–breeding mosquito species, representing about 89% of the mosquito larvae and pupae collected from water-filled tree holes and artificial containers at all sites during the

Table 1. Total number and percent abundance of immature mosquitoes (larvae and pupae) collected from tree holes and artificial containers at 3 study sites in Boone County, Missouri, May–October, 1986–88.

Mosquito species	Tree holes	Artificial containers
	No. collected (%)	No. collected (%)
Aedes triseriatus	34,514 (88.6)	14,422 (89.5)
Orthopodomyia signifera	2,355 (6.1)	887 (5.5)
Anopheles barberi	1,123 (2.9)	507 (3.1)
Aedes hendersoni	854 (2.2)	47 (0.3)
Toxorhynchites rutilus sepentrionalis	65 (0.2)	18 (0.1)
Culex restuans	0	192 (1.2)
Culex territans	0	44 (0.3)
Psorophora ferox	2 (0.01)	0
Totals	38,909	16,117

3 seasons of 1986–1988 (Table 1). The immatures of *Ae. triseriatus* were found in water-filled tree holes of all 6 tree species and all water-filled artificial containers sampled during each season. *Orthopodomyia signifera* (6%) and *An. barberi* (3%) were the 2nd and 3rd most abundant species from all the habitats sampled. Larvae and pupae of *Cx. restuans* (n = 192) and *Cx. territans* (n = 44) were collected only from artificial containers.

The 1st phase of the experiment conducted during the 1986 summer season involved feeding female *Ae. triseriatus* with canine blood infected with *D. immitis* on blood-soaked cotton pledgets placed on the screen. This was discontinued because all of the adult female mosquitoes died within 2 days after feeding. The reason for the excessive mortality was unknown, but one possibility was the chemical treatment of the blood to prevent coagulation.

The 2nd phase of the experiment included a dog naturally infected with *D. immitis* as a source of infection for the female mosquitoes during the summer seasons (June–August) of 1987 and 1988. Approximately 750 female *Ae. triseriatus* were fed on the dog, resulting in 630 infected mosquitoes (84% infection). The average numbers of 1st (L1) and 2nd (L2) larval stages of *D. immitis* found in laboratory-reared female *Ae. triseriatus* during the experimental period were approximately 9 and 11 in 1987 and 9 and 10 in 1988, respectively (Table 2). Third-stage (L3) infective larvae were 1st observed in the hemocoel, thorax, and head region on day 15 after feeding. The average number of infective larvae (L3) of *D. immitis* found in female *Ae. triseriatus* was 14.2 and 15.5 in the experimental period of June–August 1987 and 1988, respectively (Table 2). A significant difference ($P > 0.05$) was not found in numbers of infective 3rd-stage larvae found in female *Ae. triseriatus* during the 2 seasons in 1987 and 1988, suggesting that the experimental procedure was applied consistently and that this species was able to support the development of infective larvae of *D. immitis* in both seasons.

DISCUSSION

Fifty-five species of mosquitoes from a variety of habitats have been reported in Missouri (Darsie and Ward 1981, McCauley et al. 2000). In this study, only 8 mosquito species were collected from tree holes and artificial containers in central Missouri, compared with about 12 species from similar habitats reported statewide (McCauley et al. 2000). Although known in Missouri, the common artificial container–breeding mosquito *Ae.* (*Stegomyia*) *aegypti* (Linn.), a vector of yellow fever virus, was not collected during this study. *Aedes albopictus* and *Ae. japonicus* were not present in Missouri before 1988 and thus were not found during our survey. *Aedes triseriatus*, the dominant species in our study, is considered an important vector of La Crosse encephalitis virus in the upper Midwest and eastern part of the USA (Walker 1992, Barker et al. 2003), as well as a potential vector of WNV (Mans et al. 2004).

The mosquitoes collected in this study appeared to have 3 different patterns of focal distribution.

Table 2. Average number of larval stages of *Dirofilaria immitis* found in laboratory-reared female *Aedes triseriatus* that were fed on a microfilaremic dog in Columbia, MO, June–August, 1987 and 1988.

Larval stage	Days after infection	Average no. females dissected (SE)[1]	Average no. microfilarie found (SE)	
		1987 and 1988	1987	1988
First	2 and 9	7.5 (3.5)	9.3 (4.5)	8.7 (4.2)
Second	9, 13, and 15	15.0 (5.0)	10.9 (3.8)	10.4 (3.6)
Third	15 and 17	22.5 (3.5)	14.2 (2.4)	15.5 (2.6)

[1] SE, standard error.

Aedes triseriatus, Or. signifera, and *An. barberi* apparently colonized tree holes and containers equally. Although *Ae. hendersoni* and *Tx. rutilus* also occupied both kinds of habitats, they were more abundant in tree holes than in containers. The 2 *Culex* species (*Cx. restuans* and *Cx. territans*) were only present in containers. To our knowledge, this was the 1st record of the collection of *Ps. ferox* in tree holes, probably representing a highly anomalous selection of an oviposition site. The absence of *Cx.* (*Cux.*) *pipiens* Linn. was surprising, considering the extent of the collection effort. This result suggests that *Cx. pipiens* only occurs in urban habitats in central Missouri.

The method of feeding of females *Ae. triseriatus* canine blood infected with *D. immitis* through a membrane on blood-soaked cotton pledgets was discontinued because of excessive mortality of the females 2 days after feeding. The cause of excessive mortality was unknown but it could have been attributed to the possibility that heparin plus EDTA was toxic, the burden of microfilariae was too high, or the blood was improperly digested. The latter possibility seems most likely because of the abnormal expansion of the abdomens of females observed before death. Little information is recorded or available in the literature pertaining to similar results, with one exception being a study by Bemrick and Moorehouse (1968), who fed *Ae.* (*Ochlerotatus*) *vigilax* Skuse, *Cx.* (*Culex*) *annulirostris* Skuse, and *Cx.* (*Cux.*) *pipiens quinquefasciatus* Say blood infected with *D. immitis* through a membrane and obtained very low survival rate (between 0% and 2.6% of infective 3rd-stage larvae). Other related experimental transmission studies with filarial worms demonstrated that a high level of mortality occurred in the last days of filarial larval development within the mosquito. For example, Kershaw et al. (1955) and Webber and Hawking (1955) also showed that high mortality occurred during the later part of the filarial larval developmental period (10–12 days and 21–25 days, respectively, after the blood meal).

The results of our study indicate that laboratory-reared female *Ae. triseriatus* were able to support larval development of *D. immitis* when microfilariae were obtained from a blood meal from a dog. This result indicates that *Ae. triseriatus* is physiologically capable of supporting infective *D. immitis* and is a possible vector of canine dirofilariasis in Missouri. Our results also agree with those of Intermill (1973), Todaro et al. (1977), Rogers and Newson (1979), and Roberts et al. (1985), who reported larval development of *D. immitis* to the infective 3rd larval stage in *Ae. triseriatus.* However, naturally infected female *Ae. triseriatus* were not found during our survey. Experimental dog-to-dog transmission with wild-caught female *Ae. triseriatus* is still required before the precise vector role of this species can be assessed fully for Missouri or any other location. In addition, if *Ae. triseriatus* is capable of transmitting microfilariae of *D. immitis,* its abundance and broad distribution would make it an important vector of dog heartworm disease in Missouri (Smith 1967, McCauley et al. 2000). Our observations that *Ae. triseriatus* was the most abundant tree hole– and artificial container–breeding mosquito in the summer seasons of 1986–1988 over a period of 6 months (May–October) in central Missouri provide an indication of the dominance of the species at that time. In our laboratory study, female *Ae. triseriatus* fed avidly on a microfilaremic dog; therefore, this species should be considered seriously by veterinary and vector control authorities concerned with dog heartworm disease.

Examination of our data suggests that *Ae. triseriatus* was the most abundant of the mosquitoes that were equally numerous in containers and tree holes. Although current data do not exist, it would be interesting to determine the subsequent effect of introduction of *Ae. albopictus* and *Ae. japonicus* in central Missouri. One question is whether the number of *Ae. triseriatus* has been reduced in either tree holes or containers by the introduced species. Another question is whether the less abundant species in these larval habitats (*Or. Signifiera* and *An. barberi*) somehow escape competitive exclusion. It is even possible that the formerly scarce species became more abundant as a result of any diminution of their presumed principal competitor, *Ae. triseriatus.* Answering these and other questions by repeating this study now that *Ae. albopictus* and *Ae. japonicus* are present could shed light on some of the potential interactions of native mosquito fauna after introduction of an invasive mosquito species.

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